THE CHEMISTRY OF STREPTOMYCIN

BY

ROBERT KINGSBURY CLARK, JR.

A. B., DePauw University, 1943

AN ABSTRACT OF A THESIS

SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF DOCTOR OF PHILOSOPHY IN CHEMISTRY
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CHAPTER I

THE CHEMISTRY OF STREPTOMYCIN

Introduction

During the past few years much attention has been given to the chemistry of the antibiotic substances. These are naturally occurring compounds produced by fungi, bacteria, and other microorganisms, which inhibit the growth of certain living organisms. The amazing clinical results achieved with penicillin therapy stimulated a search for other antibiotic substances, and particularly those active against gram negative organisms which are not attacked by penicillin.

Streptothricin and streptomycin, discovered in 1941 and 1943 respectively by Waksman and co-workers (1,2), offered promise in this direction since they exhibited properties quite different from those of penicillin. Streptothricin seemed to be less desirable antibiotic due to its toxic properties. Therefore, in our laboratories a study was undertaken of the isolation and characterization of streptomycin.

At the time of our investigation of streptothricin and streptomycin only crude preparations of streptothricin had been obtained by adsorption on charcoal and elution with dilute acid. The fact was known at this time that streptothricin was insoluble in non-polar solvents.

Also at the time of this investigation other laboratories (Merck and Co., Squibb Medical Institute, and a group under Dr. Wolfrom at Ohio State University) were doing research on these two antibiotic compounds.

Discussion of Results

Streptothricin could be isolated by adsorption from the brews with 1 per cent Norite A. The carbon cake was

washed successively with water, ethanol, and methanol before the 0.1 N methanolic hydrogen chloride elution of the active fraction. Usually three elutions were made, and a crude product of streptothricin assaying 100 units/mg. could be obtained by precipitation with ether.

The carbon adsorption technique was adapted for the isolation of crude streptomycin. In this case it was necessary to lower the pH of the brews to 2.0 and add 0.5 per cent Norite A in order to clarify the beer. This process removed little activity. When the pH was adjusted with potassium hydroxide to 8.0, 1 per cent Norite A removed the majority of the streptomycin. Again the carbon cake was washed with water, ethanol and then methanol before the three 0.1 N methanolic hydrogen chloride elutions. The three latter elutions were immediately precipitated with ether, yielding a tan amorphous powder assaying 100-300 units/mg.

This solid was further purified by chromatography over an acid washed alumina column in 70 per cent methanol. The most active fractions obtained assayed 800 units/mg., and were easily converted into the crystalline calcium chloride double salt of streptomycin reported by Folkers (3). It had been found that streptomycin contained a substituted guanidine group, and the Sakaguchi test along with the chloride test aided in determination of the active material in the chromatogram eluates, as pure streptomycin is a colorless material.

With 0.1 N methanolic hydrogen chloride streptomycin was inactivated in 24 hours, and two degradation products, streptidine (a guanidine) and methyl streptobiosaminide dimethyl acetal hydrochloride, were separated by addition of 1 volume of ether, which precipitated the amorphous streptidine hydrochloride.

With picric acid and streptidine hydrochloride, the crystalline streptidine picrate was isolated, and this could be converted to streptidine sulfate by adding a sufficient

volume of 6 N sulfuric acid and precipitating the crystalline sulfate with acetone.

Streptidine sulfate was hydrolized with lithium hydroxide to one mole of a new base (streptamine), two moles of carbon dioxide and four moles of ammonia. Streptamine was isolated as the sulfate, and the N,N-dibenzoylstreptamine prepared by hydrolysis of the O-benzoyl groups from poly-benzoylstreptamine with methanolic sodium hydroxide.

Periodate studies were made on streptidine, streptamine and N,N-dibenzoylstreptamine. Streptidine consumed two moles of sodium periodate, streptamine six moles of periodate and N,N-dibenzyl streptamine two moles of periodate. From these data, it was concluded that streptamine is a diamino-tetrahydroxy-cyclohexane. The guanido and N-benzoyl groups must be either in the 1,3 or 1,4 positions, since the 1,2 isomers would consume three moles of sodium periodate As the amount of formic acid produced during this reaction was less than 1 mole, it was necessary to isolate the oxidation product. Another member of the antibiotic group in this laboratory isolated the dialdehyde and by means of bromine water oxidation converted it to α,α'-dibenzamino-β-hydroxy glutaric acid. Therefore, streptidine is the 1,3-diguanido-2,4,5,6-tetrahydroxycyclohexane.

The other fragment was isolated and purified by chromatography. This fragment is optically active. With pyridine and acetic anhydride the crystalline tetraacetyl methyl streptobiosaminide dimethyl acetal was isolated. The N-acetyl methyl streptobiosaminide was obtained by saponification with dilute sodium hydroxide. On a periodate study this consumed one mole of sodium periodate immediately, as would be expected on the basis of Kuehl's formula (4).

On methanolic sodium hydroxide hydrolysis of streptomycin, maltol and streptidine were isolated and characterized.

Summary

A procedure for the isolation of crude streptomycin and streptothricin has been devised.

An excellent method has been devised for the purification of crude streptomycin. The process involves the passage of crude streptomycin hydrochloride in aqueous methanol over an acid washed alumina column. The peak fractions from the column give almost pure streptomycin from which the crystalline calcium chloride double salt has been prepared.

Streptomycin has been degraded to a guanidine containing fragment (streptidine) and a non-guanidine containing fragment (methyl streptobiosaminide dimethyl acetal) by the action of methanolic hydrogen chloride.

Streptidine has been hydrolized to streptamine, ammonia and carbon dioxide by alkalis and through a series of periodate studies on streptidine, streptamine and N,N-dibenzoylstreptamine, the structure of streptidine has been established as 1,3-diguanido-2,4,5,6-tetrahydroxycyclohexane.

Methyl streptobiosaminide dimethyl acetal has been converted to the tetraacetyl and N-acetyl derivatives. Periodate studies on N-acetyl streptobiosamine dimethyl acetal substantiates Kuehl's formula for this fragment.

Streptomycin yields streptidine and maltol on treatment with methanolic alkali.

BIBLIOGRAPHY

1. Waksman, S. A., Woodruff, H. B., and Horning, E. S., *J. Bact.*, **42,** 816 (1941).
2. Schatz, A., Bugie, E., and Waksman, S. A., *Proc. Soc. Exptl. Biol. Med.*, **55,** 66 (1944).
3. Peck, R. L., *et al.*, *J. Am. Chem. Soc.*, **67,** 1866 (1945).
4 Kuehl, F A., *et al.*, *J. Am. Chem. Soc.*, **68,** 2679 (1946).

CHAPTER II

AMINO ANALOGS OF INOSITOL

Introduction

The structure of streptamine was established by various laboratories (1,2,3) as 1,3-diamino-2,4,5,6-tetrahydroxycyclohexane. Since streptamine is optically inactive and does not appear to be resolvable, it has been assigned a *meso* configuration. There are eight possible *meso* forms of streptamine. Hence, a synthetic approach to this substance affords little promise. Nevertheless, it seemed of interest to prepare mono- and diamino analogs of inositol and study their properties as an approach to the synthesis of streptamine itself. Such a program was undertaken in this laboratory and certain of the results are described in this thesis.

Inositol, which is hexahydroxycyclohexane, is a compound which one would obviously investigate on such a problem.

Fulmer and Underkofler (4) reported a diketoinositol obtained by the action of *Acetobacter suboxydans* fermentation on *meso*-inositol. This result was in contrast to the report of Kluyver (5) and Posternak (6) who reported the isolation of *scyllo-meso*-inosose (a monoketoinositol) from such a fermentation by *A. suboxydans.*

Posternak (7) also had prepared a monoketoinositol (*dl-epi-meso-inosose*) by nitric acid oxidation of *meso*-inositol and found it to be different from the *scyllo-meso*-inosose obtained by *A. suboxydans* oxidation of *meso*-inositol.

Chargaff (8) made studies in the Warburg apparatus on the oxidation of various inositols by a resting cell suspension of *A. suboxydans.* Of interest to our group was the report that Posternak's *dl-epi-meso*-inosose consumed a half atom of oxygen per mole of compound. *d*-Inositol, *l*-inositol, *meso*-inositol, and quercite also were oxidized.

From these and other reports Posternak (9) formulated

the grouping that was necessary for oxidation of a cyclitol by *A. suboxydans*. He found that there must be at least two adjacent hydroxyl groups with a *cis* configuration in order that the enzyme system would oxidize one of the hydroxyl groups to a ketone group.

Discussion of Results

Since the ketoinositol of Fulmer and Underkofler (4) was thought to be a diketoinositol, it seemed possible that this compound might be a starting material for a diamino analog of inositol, particularly if the diketone should be a 1,3 diketone.

From our cultures of *A. suboxydans* (A. T. C. C. No. 621) only *scyllo-meso*-inosose was obtained and in high yields from a five day fermentation. The decrease in inositol was determined by a yeast assay and at the end of three days, no inositol remained. The identity of the inosose was confirmed by preparation of the phenylhydrazone, and the two penta-acetates, and by reduction to scyllite.

Since another part of our group was unable to carry out the oxidation of *dl-epi-meso*-inosose by *A. suboxydans* on a large scale, it was considered more promising to attempt to convert the ketone group of *dl-epi-meso*-inosose to the amino group. The conditions of the reduction were worked out with *scyllo-meso*-inosose phenylhydrazone.

The amine derived from the high pressure reduction of *scyllo-meso*-inosose phenylhydrazone with hydrogen and Raney nickel was isolated as the free amine. Because the configuration of this amine is unknown at this time it will be designed as Amine-Sc throughout the remainder of this thesis. The hexaacetyl-Amine-Sc, N-benzoyl-Amine-Sc, and Amine-Sc hydrochloride were prepared and characterized.

With Raney nickel at 1500 pounds of hydrogen, *dl-epi-meso*-inososephenylhydrazone was reduced to the amine. As in the case of Amine-Sc, this amine will be designated as

Amine-Ep until its configuration has been established.

As this amine is somewhat soluble in methanol and also is difficult to obtain crystalline by precipitation with ether, the Amine-Ep was isolated as the crystalline hexaacetate.

With hydrochloric acid hydrolysis, the Amine-Ep hydrochloride was prepared with methanol saturated with ammonia the hexaacetyl Amine-Ep was converted to the N-acetyl derivative.

The action of resting cells of *A. suboxydans* on these various derivatives was determined by the oxygen consumption in the Warburg apparatus. *Scyllo-meso*-inosose, Amine-Sc, N-acetyl-Amine-Sc, scyllite, N-acetyl-Amine-Ep were not oxidized by the enzyme system. *dl-Epi-meso*-inosose, and Amine-Ep hydrochloride were oxidized.

The compounds that were not oxidized by *A. suboxydans* were added to the cell suspension and inositol in order to determine if they might be antimetabolites for inositol. They were found to have no inhibitory effect on the oxidation of inositol.

Summary

From a five day culture of inositol and *A. suboxydans* a high yield of *scyllo-meso*-inosose was obtained. This product was characterized by its phenylhydrazone and hexaacetyl derivatives, and by reduction to scyllite and the isolation of hexaacetyl-scyllite and scyllite.

Scyllo-meso-inosose phenylhydrazone and *dl-epi-meso*-inosose phenylhydrazone were reduced with hydrogen and Raney nickel at 1500 pounds pressure and 130°C. to the amino analogs of inositol.

Amine-Sc was isolated as the free amine and was characterized by its hexaacetyl, N-acetyl, N-benzoyl, and hydrochloride derivatives.

The Amine-Ep was isolated as the hexaacetyl derivative. This was converted to its hydrochloride and N-acetyl derivatives.

In the Warburg apparatus with resting cells of *A. suboxydans,* the oxidative action on *scyllo-meso*-inosose, *dl-epi-meso*-inosose, Amine-Sc, N-acetyl-Amine-Sc, Amine-Ep hydrochloride, N-acetyl-Amine-Ep, and scyllite were tested. Only *dl-epi-meso*-inosose and Amine-Ep hydrochloride were oxidized by *A. suboxydans.*

The compounds which were not oxidized by *A. suboxydans* under the Warburg conditions were not antimetabolites for inositol, because the inositol consumed the same amount of oxygen in their presence as in their absence.

BIBLIOGRAPHY

1. Carter, H. E., *et al., Science,* **103,** 540 (1946).
2. Fried, J., Boyack, G. A., and Wintersteiner, O., *J. Biol. Chem.,* **162,** 391 (1946).
3. Peck, R. L., *et al., J. Am. Chem. Soc.,* **68,** 772 (1946).
4. Fulmer, E. I., and Underkofler, L. A., *Iowa State College J. Sci.,* **16,** 120 (1947).
5. Kluyver, A. J., and Boezoardt, A. G. J., *Rec. Trav. Chim.,* **58,** 956 (1939).
6. Posternak, T., *Helv. Chim. Acta,* **25,** 746 (1942).
7. *ibid.,* **29,** 1991 (1946).
8. Chargaff, E., *J. Biol. Chem.,* **165,** 379 (1946).
9. Posternak, T., *Helv. Chim. Acta,* **30,** 441 (1947).

VITA

The author was born in Peoria, Illinois, March 18, 1922. He attended the public schools, and upon graduation from Peoria Central High School, entered DePauw University in September, 1940. In September, 1941, he was awarded a Rector Scholarship for the remainder of his undergraduate college education. He received his A. B. degree from DePauw University in December, 1943, having completed his requirements in August of that year.

In October, 1943, he entered the University of Illinois as an unclassified student. In February, 1944, the author entered the Graduate College of the University of Illinois. The author held research appointments in chemistry for periods of October, 1943, to February, 1947, and July, 1947, to February, 1948. He held teaching assistantships in chemistry from September, 1946, to June, 1947, and from September, 1947, to February, 1948. He is a member of Sigma Xi and Phi Lambda Upsilon.